Chapter: 8

Summary and Conclusion

Introduction

An increasing reliance on the use of “naturals” of plant origin, even in the industrialized societies has been traced to the development of several drugs and chemotherapeutics from the medicinal plants. The use of herbal preparations from plants in the form of local medicine dates back to 4000-5000 B.C. Since olden days, plants or their parts have been used to treat many ailments, and India, with approximately 45,000 plant species in its vast plant wealth, seems to include several thousand plants which have been claimed to possess medicinal properties. In spite of the tremendous advances made in the field of modern medicine, there are still a number of ailments for which suitable drugs are not available. Diabetes Mellitus is one among those and nowadays it is a global problem. Diabetes Mellitus was known to ancient scholars of Ayurveda some 3000 years back. The association of frequent urination with a sweet tasting (and ants attracting) substance in the urine was first time reported in Charaka Samhita, the ancient Ayurvedic classic. India accounts for the largest number of people (50.8 million) suffering from
diabetes in the world. India continues to be the “diabetes capital” of the world, and by 2030, nearly 9 per cent of the country’s population is likely to be affected from the disease. The most common conventional treatment for diabetes is insulin which has prominent side effects. Neither insulin nor other modern pharmaceuticals have been shown to modify the course of diabetic complications. Many natural products and herbal medicines have been recommended for the treatment of diabetes. Plants are being used heavily to treat diabetes mellitus, an effort that resulted in more than 700 recipes containing more than 400 plants known for their antidiabetic activity. DiaMedBase, a diabetes literature database of medicinal plants includes 742 records, consisting of about 309 genera and 389 species of plants, described to possess medicinal properties against diabetes. India is facing an explosive increase in the incidence of diabetes mellitus and, therefore, there is an urgent need to find cost effective, if possible, indigenous and safe drugs for diabetes. An ideal antidiabetic drug will be one which lowers the raised blood glucose to normal level without causing hypoglycaemia in any state of nutrition. In addition, modern therapies are far too costly to be used by the majority of diabetes sufferers from relatively poorer nations.

Keeping this in mind the present investigation aims to scientifically screen study and evaluate one of the plants, Costus pictus D. Don, as a potential source of an effective, affordable, easily available herbal medicine for diabetes mellitus. Since this is a recently introduced plant in India, in the state of Kerala, investigations have been carried out to confirm the morphological, molecular, biochemical, phytochemical and pharmacological characteristics of the plant. Detailed research on the chemistry and pharmacology of products of this plant are also essential and this may eventually lead to the discovery of the “magic” medicine that can be used in the treatment of diabetes. The present study was, therefore, undertaken with the following objectives.

1. To verify the survival capacity of the herb (3 clones (accessions) collected from different parts of Kerala) in quite different agroclimatic zones of India by cultivating them in Sitapur, U.P, and in Nainital, Uttarakhand. If the plant grows well under these contrasting conditions, it will be a good indication and suggestive of the fact that it can perhaps be cultivated widely in India, which is dreadfully affected with diabetes mellitus.
2. Standardize propagation protocols for this plant using stem cuttings.

3. Isolation of DNA for molecular analyses to find the genetic diversity, if any, in the plants originally collected from different regions of Kerala.

4. Extraction, isolation, separation and purification of major phytochemicals present in the rhizome, stem and leaves followed by fractionation and screening for the presence of active compounds.

5. Evaluation of hypoglycaemic activity of the crude plant extracts and isolated compounds following standard assays.

Cultivation and morphological analysis of *Costus pictus*

*Costus pictus* D. Don. (Costaceae), a perennial herb, is a wonderful and well known medicinal plant owing to its antidiabetic property, hence commonly called as the ‘insulin plant’. Although only recently introduced in India, it is widely cultivated in south India and has also started to appear as wild in many places. It is a recent introduction to India from Mexico during 2002-03, and it is normally grown in home gardens as an ornamental plant, especially in Kerala, the state where it was first introduced (Merina, 2004). The occurrence of *C. pictus* in northern part of India is unknown. The wide spread popularity of this plant in south India is due to its pronounced antidiabetic activity (Sabu, 2006). Since it is a newly introduced plant, investigations have to be made to verify the survival capacity of the plant in different agroclimatic zones of India. For this purpose, the objective of this chapter is designed to evaluate survival and growth of the plant by cultivating in Sitapur, U.P. and Nainital, Uttarakhand. Additional plants of three clones were also maintained in pots kept in the green house of GBPIHED, Kosi-Katarmal, Almora, Uttarakhand.

The plants were collected from three different locations of Kerala (south, middle and north) and attempts were made to cultivate it at Nainital, Almora and Sitapur. It was found to acclimatize under outdoor conditions in the north Indian plains at Sitapur but not at high mountain ranges like Nainital and Almora, where the winter temperatures are very low. The rhizome pieces that were planted at the D.S.B. Campus of Kumaun University, Nainital did not show any sign of growth, may be due to very low temperatures
experienced during winter months. However, the three clones maintained in the green house of GBPHIED, Almora showed good growth.

India is famous for its varying climatic conditions and this tropical plant, C. pictus, is well suited to such conditions, except in regions with extreme winter. The results of growth parameters taken on plants grown at Sitapur clearly show that C. pictus can grow well under areas with full sun as well as under slight shade with well draining loamy soil. However, during winter months (October to March) the plant shows minimal growth, which may be considered as a resting (dormant) period for the plant under conditions prevalent at Sitapur during winter. The plant showed maximum increment in growth parameters, such as the number of clumps per plant, height of clump (stems) during April to October. It would seem fair to conclude that the warmer conditions favour plant growth, and it is not possible to cultivate it in low temperature areas like Nainital and Almora under natural field conditions.

The morphological analysis of three clones collected from three locations in Kerala did not show any significant difference in their vegetative as well as reproductive characters. A total of 54 morphological/growth parameters were recorded for all the 3 clones (4 plants per clone) collected from different locations of Kerala, and the results were found to be same in respect of various parameters. From this it is concluded that the 3 clones (or accessions) are one and the same. The plant being a recent introduction (Merina, 2005) to Kerala, there is little possibility of any variation occurring in morphological parameters in such a short time frame. The results of this chapter indicate the possibilities of its cultivation in different, warmer and humid parts of India so as to exploit its utility as a source of antidiabetic biomolecules.

Studies on the vegetative propagation of Costus pictus

In the 3rd chapter evaluation of propagation protocols of for C. pictus has been carried out, using 6 plant growth regulators. C. pictus regenerates by rhizome as well as through stem cuttings. Since the plant is subjected to severe winter conditions in north India and perennates through underground parts for some duration in the year, the period of growth is confined from spring to autumn through summer and rainy months, i.e., April to November. The improved vegetative multiplication can help in promoting large scale
propagation for cultivation of this species. Plant growth regulators (PGRs) are important during the whole process of growth and development of plants (Davies, 1987). The current study was attempted to evaluate the impact of PGRs in the mass propagation of *C. pictus*. In the present study, the selected plant growth regulators (chemicals) did not show much differences on various parameters; somewhat stimulatory influence was observed on shoot characteristics by IBA (auxin) and BAP (cytokinin) treatments at both the concentrations used (10 μM, 100 μM). The IBA and BAP treatments exhibited maximum values in terms of sprouting of axillary buds, number of clumps (shoots) and the overall shoot length. The effect of used plant growth regulators/chemicals showed negligible differences on leaf characteristics. IBA (10 μM), BAP (100 μM) and KNO₃ (5 mM) produced maximum number of leaves, while GA₃ and ABA (10 μM) were less effective. The results of average rhizome weight, diameter and number of roots, etc. did not show much difference among various treated group of plants. The IBA (10 μM), BAP (10 μM) and KNO₃ could stimulate the growth of rhizome and roots compared to untreated control group of plants, as also in the shoot and leaf related parameters. The influence of PGRs on biomass of *C. pictus* has also been assessed and reported. Results showed that KNO₃ had maximum stimulatory effect on biomass (above and below ground, and the total biomass) production, while minimal biomass production was seen in ABA treated group. It would seem safe to say that the plant naturally possess excellent ability to propagate vegetatively, and by used large chemicals & PGRs could not show any major effect, and are, thus, not recommended for use in the nursery practices for *C. pictus*.

The overall response of selected PGRs and chemicals on the growth of *C. pictus*, stimulatory effect was observed with KNO₃, IBA and BAP. On the other hand, minimal growth was observed in GA₃, ABA and Bavistin treated groups of plants. This negative impact on growth might be due to the inhibitory effect of these PGRs on vegetative growth. These preliminary findings should pave the way for future work on improvement, conservation and mass propagation of *C. pictus*, to fulfill the ever increasing demand for commercial level cultivation of this important antidiabetic plant.
Molecular and physiological analyses of *Costus pictus*

The investigations on molecular and physiological characteristics of *C. pictus* were also carried out during the study. Since *C. pictus* is a newly introduced plant, any reference regarding its molecular analysis is so far not available. In the study, *C. pictus* plants, originally collected from different parts of Kerala, and grown in the green house at GBPIHED, Almora were profiled using RAPD markers to assess genetic diversity, if any. In RAPD analysis during the primer-screening step, out of the 40 random primers used, 10 primers produced clear and scoreable amplification products in different samples of *C. pictus*. The amplification profiles of total genomic DNA from *C. pictus* plants, with ten selected primers produced a total of 44 fragments ranging in size from 0.3-2.9 kb, out of which 4 (9.09%) were polymorphic. The number of fragments produced by a primer ranged from 2 (OPA10 & OPC 19) to 6 (OPA 3, OPA 11 & OPC18). Primer OPA 2 produced 4 amplified products. None of them were polymorphic.

Patterns of RAPD fragments produced by OPA3 and OPA7 showed that decamer primer OPA3 produced 6 amplified fragments out of which 2 bands were polymorphic. Size of these products was 0.6 to 2.9 kb. Primer OPA7 produced 4 bands after PCR analysis. None of them were polymorphic and ranged in size from 0.5 to 2.6 kb. Primer OPA 10 produced only two monomorphic bands. Amongst the primers used for RAPD analysis, primer OPC 5 produced 4 amplified products without any polymorphic bands. Size of the amplified product was 0.5 to 0.8 kb. OPC 16 produced a total of 5 amplified products, among them only 1 product was polymorphic. The size range was from 0.5 to 1.8 kb.

RAPD analyses did not reveal the presence of any genetic differences between the plants of *C. pictus*, originally collected from different areas. The similarity within plants was found to vary from 0.96 to 1.0. Very high similarity observed between these plants may be due to propagation by vegetative means. Further, the results of present investigation using RAPD markers may serve as reference information for breeding and germplasm conservation of *C. pictus* in the future.

The results obtained on the physiological (gas exchange) parameters suggest that the three clones do not differ and may represent the same stock. Very little difference in
physiological parameters (net photosynthesis, stomatal conductance, transpiration rate, internal CO$_2$; Fv/Fm, etc.) of the three clones suggests no genetic or ecotypic variation in the plants collected from three different locations of Kerala. It implies that the three clones represent one and the same stock. Since the plant is propagated through vegetative means, there are only limited chances to observe heritable physiological responses in *C. pictus*.

**Phytochemical analysis of Costus pictus**

Based on the reported activity of the insulin plant, *C. pictus*, Chapter 5 was designed to investigate the activity in extracts of various plant materials (leaf, stem and rhizome) prepared with various solvents, in order to screen the major phytochemicals their separation (and possible identification) so as to pinpoint the active principle for more directed exploitation of the plant in antidiabetic therapeutics. Phytochemical screening was performed using standard procedure like extraction, separation and identification of the plant constituents. The moisture content was calculated to be more than 85% in all three materials, *i.e.*, leaf, stem and rhizome, based on the difference observed in fresh and dry weight of three samples of *C. pictus*. This appears to be a common character shared by all the members of Costaceae and Zingiberaceae.

For extraction two methods (maceration and hot continuous extraction) were tried with all three samples (rhizome, stem and leaf) using 4 solvents; this resulted in a total of 24 extracts. Maximum yield of the extract was obtained from leaf sample using methanol as a solvent through the maceration method. The plant extracts are an attractive source of new drugs and have shown promising results for the treatment of diabetes. The preliminary phytochemical qualitative tests for steroid, phenols etc. conducted with all 24 (12+12) extracts prepared using 2 extraction methods revealed similarity in respect of the presence of these phytochemicals in various extracts. The results of TLC analysis of all 24 extracts also indicated great similarity in the chemical constituents. However, methanolic leaf extract of *C. pictus* was found to have maximum concentration of secondary metabolites. This is in broad agreement with previous studies (Jothisel et al., 2009; Gireesh et al., 2009; Kumawat and Shimpi, 2009). Therefore, methanolic leaf extract (maceration) was used for further analyses by separation, purification and
identification of compounds. The HPLC chromatogram indicated the presence of two major compounds in crude methanolic leaf extract. Among 13 peaks seen in the HPLC chromatogram, the two main peaks represented 42.27% and 39.80%, respectively of the total compounds present in the extract, and these values put together indicated that 82.07% of the extract consisted of these two compounds. Therefore, further investigations were carried out with these two major compounds.

Column chromatography also resulted in a major fraction (2), which was further analyzed with HPLC and TLC. The HPLC chromatogram of the fraction showed one peak (99.7530%) and it matched with the results of TLC analysis. The colour of the major spots under UV light and spraying with ammonia reagent indicated the presence of glycoside in the extract. Therefore, it was inferred that the major phytochemical found in the crude methanolic leaf extract could be one of several glycosides.

The same second fraction of column chromatography was subjected to GC-MS and the spectral graphs (Mass spectrometric fragmentation patterns) of the sample and the reference (marker) compound were found to have somewhat similarity in their fragmentation pattern, at least up to M⁺ 116. β-L- Arabinopyranose methyl glycoside was used as reference compound. Based on these results it was concluded that the major glycoside in the sample could be β- L- Arabinopyranose methyl glycoside; the identification is at best only tentative. This compound may also be responsible for the antidiabetic activity; however, much work is required to confirm this.

From the available literature on various studies of C. pictus, it seems that there are no reports on the comparative, systematic studies related to the activity of various extracts. Therefore, continuing the investigations further, the antidiabetic activity of C. pictus, was assessed by way of looking at the hypoglycemic activity of various extracts and fractions prepared from the leaves in albino mice through OGTT (Oral glucose tolerance test); this was followed by evaluation of the antidiabetic activity of most active extract in streptozotocin induced hyperglycemic mice.

**Hypoglycaemic investigations of extracts of Costus pictus**

Due to reported effectiveness and efficacy of the plant, C. pictus, numerous studies are being conducted in various parts of the world. From the available literature on
C. pictus, there are, so far, no reports on comparative and systematic investigations related to hypoglycaemic activity of various extracts and fractions. Therefore, the present study was directed to assess hypoglycemic activity of various extracts and fractions from leaves of C. pictus in albino mice through OGTT (Oral glucose tolerance test), and evaluation of the antidiabetic activity of most active extract in streptozotocin induced hyperglycemic mice.

Highly significant (p< 0.001) and promising antihyperglycaemic effect (OGTT) was obtained in 13 extracts prepared in different solvents, and applied in two doses to glucose fed mice. The response of 10 extracts (except fresh juice (FJ), water crude (WC) and hexane crude (HA) extracts) on lowering of glucose levels in blood was comparable to control. The results of OGTT revealed the potency of C. pictus leaf extracts in the reduction of elevated blood glucose levels to near normal level in fasted animals. The results of OGTT showed that the improvement in respect of glucose tolerance effect could be seen within 15 minutes of oral administration of the effective extracts. Although the gradual reduction in the glucose level was observed, the maximum reduction was recorded only after 120 minutes of extract administration. The methanolic crude leaf extracts (MC, M2) at 500 mg/kg body weight (body weight) were shown to have the most pronounced effect (p< 0.001) comparable to control group in OGTT. The ethyl acetate extract (500 mg/kg body weight) was also shown to exert significant influence (p< 0.001) in lowering of blood glucose levels in glucose fed mice. MEA (500 mg/kg body weight) substantially reduced the glucose level after 90 minutes of oral administration. The effect of these three different extracts was found to be dose and time dependant.

Methanol and ethyl acetate extracts at 200 mg/kg, 500 mg/kg body weight produced the antidiabetic effect on streptozotocin injected mice, and the effect was significant at p< 0.001 compared to diabetic control group of mice. Even though, the antihyperglycaemic effect of the extract of C. pictus leaves has been studied, none of the studies have reported on the effect seen in streptozotocin induced mice. Moreover, not a single study was found regarding the evaluation of the effect of various extracts and fractions on OGTT in mice and subsequent assessment of antidiabetic activity of the extract with best activity in streptozotocin induced mice. The magnitude of reduction in blood glucose level in treated diabetic mice was found to be time and dose dependent.
The ethyl acetate extract exhibited optimal antidiabetic effect in normal mice at a dose of 500 mg/kg body weight with no contraindications. However, methanol extract exerted a more rapid antidiabetic effect at 500 mg/kg body weight by reducing the blood glucose level up to 55.5%, at 3rd week after extract administration.

The methanolic leaf extract showed maximum effect in both OGTT and antidiabetic experiment. Body weight, which is yet another important parameter in diabetes, was also found to increase with the treatment of methanol and ethyl acetate extracts. During the 21-days of experimental period, the body weight was found to reduce in diabetic mice, whereas, there was a significant gain in body weight in treated mice (p<0.001). This study also reports the effect of *C. pictus* extracts on important biochemical parameters with respect to diabetes in streptozotocin induced mice model. Serum glucose, triglycerides, total and direct bilirubin, creatinine and the liver enzymes (ALP, ALT, ASP), and total protein levels were compared in control and treated groups (p<0.001). The administration of 500 mg methanol and ethyl acetate extracts of leaves of *C. pictus* were able to bring back these biochemical parameters close to normal levels in treated mice. The overall results of antidiabetic experiments suggest that *C. pictus* leaf extract has antihyperglycemic effect, and that it protects the liver from the complications of diabetes by improving body weight as also the levels of various biochemical parameters in blood serum. However, longer duration studies on chronic models are necessary to elucidate the exact mechanism of action, so as to develop it as a potent antidiabetic drug.
Conclusions

- The plants were originally obtained from three different locations of Kerala and planted at Nainital, Almora and Sitapur. The plants could not acclimatize under outdoor conditions and failed to survive in the north Indian high mountain ranges like Nainital and Almora, but exhibited good growth and survival in the plains, at Sitapur. The plants failed to tolerate the low winter temperatures in the hills. The rhizome pieces that were planted in D.S.B. Campus of Kumaun University, Nainital did not show any signs of growth due to very low temperature during winter months. But all accessions (clones) maintained in the Institute greenhouse at GBPHIED, Kosi-Ktarimal, Almora, showed excellent survival and growth. The plant is affected by severe winter conditions in north India, and even at places like Sitapur it perennates through underground parts for some duration in the winter, and the period of growth is confined from spring to autumn, through the summer and rainy months, i.e., April to November. It would, therefore, seem fair to conclude that the warmer conditions favour plant growth, and it is not possible to cultivate it in low temperature areas like Nainital and Almora under natural field conditions. In the morphological analysis, the three clones (accessions) collected from three locations in Kerala did not show any significant difference in the 54 morphological attributes and growth parameters examined. From this it is concluded that the three clones (or accessions) are perhaps one and the same.

- In the overall response of selected plant growth regulators and chemicals on the growth of *C. pictus*, slight stimulatory effect was observed with KNO₃, IBA and BAP. On the other hand, minimal growth was observed in GA₃, ABA and Bavistin (a synthetic and systemic fungicide, with reported auxin like activity in some cases) treated groups of plants. This negative impact on growth might be due to the inhibitory effect of these plant growth regulators on the vegetative growth of *C. pictus*. These preliminary findings should pave the way for future work on improvement, conservation and mass propagation of *C. pictus*, to fulfill the ever increasing demand for commercial level cultivation of this important antidiabetic plant. It needs to be mentioned that the plant can be propagated
conveniently, without PGR/chemical treatment, by clonal & conventional methods.

- The investigations on molecular and physiological characteristics of *C. pictus* plants, collected from different parts of Kerala, India and grown in the greenhouse at GBPIHED, Almora did not reveal any significant differences among three accessions. RAPD analysis did not reveal any genetic differences between the samples of *C. pictus*. Very high similarity observed between these plants may be due to propagation by vegetative means, and that the three accessions were from one and the same original stock. RAPD analysis is capable of revealing appreciable levels of polymorphism in plants, if the samples are from genetically diverse stock. The results of present investigation using RAPD markers for studying the genetic diversity and relationship of *C. pictus* plants selected from different areas in Kerala, India may serve as reference information for further work on *C. pictus*.

- The results obtained on some physiological, namely gas exchange parameters suggest that the three clones do not differ and may represent one and the same stock. Very little difference in physiological parameters (net photosynthesis, stomatal conductance, transpiration rate, internal CO$_2$, Fv/Fm, etc) of the three clones suggest lack of any genetic or ecotypic variation in the plants collected from three different locations of Kerala. It implies that the three clones may be originally from the same stock. Moreover, the plant is propagated mainly through vegetative means, thus there are only limited chances to observe heritable physiological responses in *C. pictus*, particularly so soon after introduction in India.

- The phytochemical analysis (extraction, separation and tentative identification) of *C. pictus* extracts revealed that at least a major phytochemical found in *C. pictus* could be a glycoside (possibly similar to β-L- Arabinopyranose methyl glycoside); this may provide some lead towards the identity of the active antidiabetic compound of *C. pictus*. Further detailed studies are required in this direction.
The administration of 500 mg/kg body weight of methanol and ethyl acetate leaf extracts of *C. pictus* were able to reduce the raised blood glucose level, improve body weight, bring back blood serum biochemical parameters close to normal level in treated mice. The overall results of antidiabetic experiments suggest that *C. pictus* leaf extract contains antihyperglycemic effect, and that it protects the liver from the complications of diabetes by improving body weight and the levels of biochemical parameters in blood serum. However, longer duration studies on chronic models are necessary to elucidate the exact mechanism of action, so as to develop it as a potent antidiabetic drug.